Functional, spectroscopic and structural properties of haemoglobin from chamois (*Rupicapra rupicapra*) and steinbock (*Capra hircus ibex*)

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The functional and spectroscopic properties of chamois (*Rupicapra rupicapra*) and steinbock (*Capra hircus ibex*) haemoglobin (Hb) have been studied with special reference to the action of allosteric effectors and temperature. Moreover, the amino acid sequences of the N-terminal segments of the α - and β -chains have been determined. The present results indicate that chamois and steinbock Hbs display a low affinity for O₂, which appears to be modulated *in vivo* by Cl⁻ ions rather than 2,3-bisphosphoglycerate. The Bohr effect for O₂ binding to chamois and steinbock Hb is higher than for reindeer and bovine Hbs, being similar to that of human Hb. Moreover, the temperature-dependence of oxygenation appears intermediate between that of

human and reindeer Hbs. E.p.r. and absorption spectroscopic properties of the ferrous nitrosylated derivative of chamois and steinbock Hbs suggest that both haemoproteins are in a low-affinity conformation even in the absence of IpsP_6 . The reduced effect of polyphosphates on the functional and spectroscopic properties of chamois and steinbock Hb agree with amino acid differences in the N-terminal segment of the β -chains (i.e. the deletion of Val(NA1) and the replacement of His(NA2), present in human Hb, and Gln(NA2), present in horse Hb, by Met). The molecular mechanism modulating the basic reaction of O₂ with chamois and steinbock Hb may be linked to specific physiological needs related to the high-altitude habitats of these two animals.

INTRODUCTION

Haemoglobins are characterized by a number of complex regulatory mechanisms operative at the molecular level and designed to fulfil the physiological requirements of a given species. These mechanisms are based on the thermodynamic connection between the binding of heterotropic effectors (protons, CO₂, Cl⁻ ions and organic phosphates) and the reaction with O₂. Another important feature of the reaction of Hbs with O₂ is its temperature-dependence which is governed by the associated overall enthalpy change (Antonini and Brunori, 1971).

Ruminant Hbs are almost insensitive to the presence of organic phosphates, demonstrating an intrinsically low O₂ affinity (Perutz and Imai, 1980; di Prisco et al., 1991). In some ruminants, Cl⁻ ions, at physiological concentration, may completely replace Gri(2,3)P₂ in regulating O₂ affinity of Hb (Giardina et al., 1990a). Moreover, with respect to the effect of temperature, a very interesting example is that recently reported for some arctic ruminants, with Hb with oxygenation enthalpy that is much less exothermic than that generally reported for mammalian Hbs. This feature has been related to the mechanism of adaptation to the very cold environment in which they live. In fact, a markedly reduced oxygenation enthalpy may be particularly useful in the arctic habitat as it may allow a normal O₂ supply to reach peripheral tissues even at very low temperatures (Giardina et al., 1989; di Prisco et al., 1991). The above considerations led us to extend our investigation to Hb from chamois (*Rupicapra rupicapra*) and steinbock (*Capra hircus ibex*) because their habitats

are characterized by both high altitudes and low temperatures. The data obtained provide useful information on the structural and functional evolution of Hb in vertebrates.

MATERIALS AND METHODS

Oxygenated Hb samples from chamois (*Rupicapra rupicapra*) and steinbock (*Capra hircus ibex*) were prepared as follows (Antonini and Brunori, 1971; Giardina et al., 1990a, 1992a). Fresh blood samples from these two animals were obtained from Centro Studi di Fauna Alpina, Parco Nazionale del Gran Paradiso, Noasca (TO), Italy. The blood samples were collected into an iso-osmotic NaCl solution containing 2 mM EDTA. After centrifugation at 1000 g, supernatant was discarded and erythrocytes were washed three times with iso-osmotic NaCl by centrifugation at 1000 g for 10 min. The packed cells were lysed by adding 2 vol. of cold hypo-osmotic phosphate buffer. The stroma were removed by centrifugation at 12000 g for 30 min.

As judged by gel electrophoresis and N-terminus amino acid sequence analysis, chamois and steinbock erythrocytes contain only one Hb component.

The chamois and steinbock Hbs were stripped of anions and cations [e.g. (in)organic (poly)phosphates and Cl⁻] by passing the haemolysates through a Sephadex G-25 column equilibrated with 0.01 M Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl, and afterwards through a column of mixed-bed ion-exchange resin (Bio-Rad AG 501X8) equilibrated with deionized water; the Hb was kept at pH 7.4 (0.1 M Hepes buffer) (Giardina and

Abbreviations used: Hb, ferrous haemoglobin; HbNO, nitrosylated derivative of ferrous haemoglobin; human Hb, human adult Hb; Gri(2,3)P₂, 2,3-bisphosphoglycerate.

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Amiconi, 1981; Riggs, 1981; Ascenzi et al., 1988, 1990, 1992; Giardina et al., 1992b).

Chamois and steinbock HbNO derivatives were obtained under anaerobic conditions by sequential addition of sodium dithionite and potassium nitrite (final concentrations 86 mM and 72 mM respectively) to the oxygenated haemoprotein solution (Ascenzi et al., 1988, 1990, 1992; Giardina et al., 1992b).

Hb concentration was determined on the basis of $\epsilon = 98 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (at 415 nm) and $97 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (at 415 nm) for the ferrous nitrosylated derivative from chamois and steinbock respectively. The millimolar absorption coefficient is expressed as ϵ on a millimolar haem basis. Values of ϵ for chamois and steinbock HbNO were determined using the pyridine-haemochromogen method (Antonini and Brunori, 1971).

Gri(2,3) P_2 , Ins P_6 , Hepes, Mes and Tris buffers were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CO was obtained from Caraccioloossigeno S.r.l. (Rome, Italy). All other products were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification.

O₂-binding isotherms were determined by the tonometric method (Antonini and Brunori, 1971; Giardina and Amiconi, 1981), in the absence and presence of 3 mM Gri(2,3) P_2 between 20 and 40 °C. The overall oxygenation enthalpy at the partial pressure of the gaseous ligand at which 50% of the haem is oxygenated (δH_{50} , kJ/mol), corrected for the solubilization heat of O₂ [−12.5 kJ/mol (Antonini and Brunori, 1971)], was calculated from the integrated van't Hoff equation (Coletta et al., 1992):

$$\delta H_{50} = -19.04 \times \frac{(T_1 T_2)}{(T_1 - T_2)} \times \frac{\delta \log P_{50}}{1000}$$

where P_{50} is the partial pressure of the gaseous ligand at which 50% of the haem is oxygenated. Over the temperature range explored (20–40 °C), van't Hoff plots were linear within experimental error. Average S.D.s of $\pm 8\%$ for values of P_{50} and Hill coefficients at the partial pressure of the gaseous ligand at which 50% of the haem is oxygenated (n_{50}), and $\pm 15\%$ for δH_{50} values were calculated.

Values for the overall first-order rate constant for O₂ dissociation from the oxygenated derivative of chamois and steinbock Hb (k_{off}) were obtained in the absence and presence of 3 mM Gri(2,3) P_2 and 0.1 M Cl[−], at pH 7.4 and 20 °C, by the O₂-pulse method (i.e. by mixing a solution containing the deoxygenated derivative of Hb ([Hb] = 10 μM in haem), in the presence of sodium dithionite (86 mM), with an aqueous air-saturated buffered solution (Gibson, 1973)). An average S.D. of $\pm 8\%$ for values of k_{off} was calculated. Kinetic experiments were carried out using a Durrum–Gibson rapid-mixing stopped-flow apparatus, equipped with an Olis (Jefferson, GA, U.S.A.) analog–digital interface for real-time data reduction on a CompaQ Deskpro 286 personal computer.

Absorption spectra of chamois and steinbock Hb, in the Soret and/or visible regions, were recorded, in the absence or presence of Gri(2,3) P_2 or Ins P_6 , between 20 °C and 40 °C on double-beam spectrophotometers Kontron 860 Uvikon and Varian Cary 219.

X-band e.p.r. spectra of chamois and steinbock HbNO were recorded, in the absence or presence of Ins P_6 , at 100 K on a Bruker ESP 300 spectrometer.

Under all the experimental conditions, the ferric derivative of chamois and steinbock Hb was always lower than 3%. The concentration of the ferric derivative of chamois and steinbock Hb was determined on the basis of $\epsilon = 185 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (at 408 nm) and $187 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (at 407 nm) respectively at pH 6.5

(0.1 M Mes buffer) for the ferric derivative. The millimolar absorption coefficient is expressed as ϵ on a millimolar haem basis. Values of ϵ for chamois and steinbock aquo-met Hb were determined using the pyridine-haemochromogen method (Antonini and Brunori, 1971).

All data were obtained between pH 6.0 and 8.0, in 0.1 M Mes and Hepes buffer systems, in the absence or presence of organic polyphosphates and Cl[−]. No specific ion effects were found using different buffers with overlapping pH values.

The amino acid sequences of the first ten residues of chamois and steinbock Hb α - and β -chains were determined as follows. Hb α - and β -chain purification was achieved by h.p.l.c.; the Hb sample was applied in several portions to a reversed-phase column (Aquapore RP300, 7 mm $\times$ 250 mm; Brownlee Laboratories), developed over 15 min with a linear gradient from 35 to 50% acetonitrile in 0.2% trifluoroacetic acid, generated in a Beckman model 340 instrument at a flow rate of 3.0 ml/min. The absorbance of the effluent was monitored at both 220 and 280 nm using a Beckman model 165 variable-wavelength detector. The α - and β -chains were alkylated with 4-vinylpyridine, and 2 nmol were loaded on to the Applied Biosystem model 470 A gas-phase sequencer, equipped with an Applied Biosystem 120 A phenylthiohydantoin-analyser for the on-line detection of phenylthiohydantoin-amino acids (Petrucelli et al., 1991; Ascenzi et al., 1992).

RESULTS AND DISCUSSION

Chamois and steinbock Hbs display superimposable reactivity, spectroscopic and structural properties (see Figures 1–4 and Tables 1 and 2). Their molecular properties are similar to those of ruminant Hb (e.g. reindeer Hb), but differ from those of human and horse Hbs (Giardina et al., 1990a).

Figure 1 shows the Bohr effect for O₂ binding to human (Antonini et al., 1982; di Prisco et al., 1991; Giardina et al., 1993), reindeer (Giardina et al., 1989, 1990b; di Prisco et al., 1991), chamois and steinbock Hbs, in 0.1 M NaCl, in the absence and presence of 3 mM Gri(2,3) P_2 at 20 °C. Over the whole pH range studied (6.0–8.0, 0.1 M Mes and Hepes buffer systems), reindeer, chamois and steinbock Hbs are characterized by a low O₂ affinity (i.e. high P_{50}) even in the absence of Gri(2,3) P_2 compared with human Hb [see Antonini et al. (1982) for comparison]. Addition of 3 mM Gri(2,3) P_2 to reindeer (see di Prisco et al., 1991), chamois and steinbock Hb samples, in the presence of 0.1 M Cl[−], results in only a slight decrease in O₂ affinity below pH 7.5, values of P_{50} for O₂ binding being essentially insensitive to this organic allosteric effector at higher pH values (Figure 1). Thus in the presence of 0.1 M NaCl [and absence of Gri(2,3) P_2], values of P_{50} for O₂ binding to reindeer, chamois and steinbock Hbs are almost superimposable on those of human Hb, in the presence of both physiological cofactors [i.e. 3 mM Gri(2,3) P_2 and 0.1 M NaCl] (Figure 1). This finding is of physiological relevance, being also supported by the low level of intraerythrocytic Gri(2,3) P_2 in ruminants, which is about ten times lower than that of human erythrocytes (approx. 0.3 mM compared with approx. 5 mM) (di Prisco et al., 1991).

The amplitude of the Bohr effect ($\delta \log P_{50} / \delta \text{pH}$; where $\delta \text{pH} = 6.0\text{--}8.0$) for O₂ binding to chamois and steinbock Hb in the presence of 0.1 M NaCl (0.43) is the same as that observed for oxygenation of human Hb, which is higher than that observed for other ruminant Hbs (Fronticelli et al., 1988; Giardina et al., 1990a, 1992a; di Prisco et al., 1991). In fact, the amplitude of the Bohr effect for O₂ binding to reindeer Hb (0.28) is about 35% less than that of human, chamois and steinbock Hb, in the absence of Gri(2,3) P_2 (see Figure 1).

Figure 1 pH-dependence of O_2 affinity ($\log P_{50}$; mmHg) for human Hb (a; $\triangle$, $\blacktriangle$), reindeer Hb (b; ∇ , $\blacktriangledown$), chamois Hb (a and b; $\bullet$, $\circ$) and steinbock Hb (a and b; $\square$, $\blacksquare$), in the absence (open symbols) and presence (filled symbols) of 3 mM $Gri(2,3)P_2$

Under all the experimental conditions, values for the Hill coefficient (n_{50}) for O_2 binding to human, reindeer, chamois and steinbock Hb ranged between 2.3 and 2.6. The continuous lines are smooth curves drawn through the experimental points. Values of P_{50} and n_{50} were obtained in 0.1 M Mes and Hepes buffer systems plus 0.1 M NaCl, at 20 °C. An average S.D. of $\pm 8\%$ was calculated for P_{50} and n_{50} values. Values of P_{50} and n_{50} for O_2 binding to human and reindeer Hb were obtained from the literature (Antonini et al., 1982; Giardina et al., 1989, 1990b, 1993). For further details, see the text.

Figure 2 pH-dependence of the overall oxygenation enthalpy (ΔH_{50} , kJ/mol) for human Hb, in the absence ($\triangle$) and presence ($\blacktriangle$) of 3 mM $Gri(2,3)P_2$, reindeer Hb (∇), chamois Hb ($\circ$) and steinbock Hb ($\square$)

Overall oxygenation enthalpy for reindeer, chamois and steinbock Hb was essentially insensitive to $Gri(2,3)P_2$. The continuous lines are smooth curves drawn through the experimental points. Values of ΔH_{50} were obtained in 0.1 M Hepes buffer system plus 0.1 M NaCl, between 20 °C and 40 °C. An average S.D. of $\pm 15\%$ was calculated for ΔH_{50} values. Under all the experimental conditions, the temperature independence of ΔH_{50} values indicates that the apparent ΔC_{p50} values are close to 0 kJ/mol. Values of ΔH_{50} for O_2 binding to human and reindeer Hb were obtained from the literature (Coletta et al., 1992; Giardina et al., 1989, 1990b, 1993). For further details, see the text.

Figure 2 shows the pH-dependence of the overall oxygenation enthalpy (ΔH_{50}) for human Hb (Giardina et al., 1993), reindeer Hb (Coletta et al., 1992; Giardina et al., 1989, 1990b), chamois and steinbock Hb in the presence of 0.1 M NaCl and/or 3 mM $Gri(2,3)P_2$. Under physiological conditions [i.e. in the presence of both $Gri(2,3)P_2$ and Cl^-], values of ΔH_{50} for chamois and steinbock oxygenation are similar to those reported for O_2 binding to human Hb, ranging between approx. -26 and

Table 1 Effect of $Gri(2,3)P_2$ (3 mM) and Cl^- (0.1 M NaCl) on overall first-order rate constants for O_2 dissociation from ligated human, reindeer, chamois and steinbock Hb (k_{off})

Data were obtained at pH 7.4 (0.1 M Hepes buffer system) and 20 °C. An average S.D. of $\pm 8\%$ for values of k_{off} was calculated.

Haemoprotein	$10^{-1} \times k_{off}$ (s^{-1})		
	Without effector	$Gri(2,3)P_2$	Cl^-
Human Hb*	2.7	4.2	4.0
Reindeer Hb*	3.3	4.4	4.9
Chamois Hb†	2.8	3.4	3.5
Steinbock Hb†	2.5	3.4	3.5

* M. Coletta and B. Giardina, unpublished work.

† Present study.

Figure 3 X-band e.p.r. spectra of horse HbNO (a), in the absence (spectrum A) and presence (spectrum B) of 0.1 M $InsP_6$, reindeer HbNO (b), chamois HbNO (c) and steinbock HbNO (d)

X-band e.p.r. spectra of reindeer, chamois and steinbock HbNO were essentially insensitive to $InsP_6$. Spectra were obtained between pH 6.5 and 7.0, in 0.1 M Mes and Hepes buffer systems plus 0.1 M NaCl, at 100 K. Setting conditions: 9.42 GHz microwave frequency; 20 mW microwave power; 0.10 mT modulation amplitude. The concentration of HbNO was about 1 mM. Spectra for horse and reindeer HbNO were obtained from the literature (Ascenzi et al., 1990, 1992). For further details, see the text.

-40 kJ/mol. On the other hand, values of ΔH_{50} for O_2 binding to Hb from arctic ruminants (e.g. reindeer, musk ox, red deer and ox) are lower, ranging between approx. -10 and -15 kJ/mol [Giardina et al. (1992a) and Coletta et al. (1992)].

Table 1 gives values of the overall first-order rate constants for O_2 dissociation from ligated human, reindeer, chamois and steinbock Hb (i.e., values of k_{off}), in the absence and presence of allosteric effectors at pH 7.4 and 20 °C. In the case of chamois and steinbock Hbs, the overall O_2 dissociation rate is affected by both Cl^- and $Gri(2,3)P_2$ to a very similar extent, a feature observed also in the case of reindeer and human Hbs (Giardina et al., 1990a). Therefore the different effects of Cl^- and $Gri(2,3)P_2$ on O_2 binding at equilibrium (see Figure 1) must mainly be exerted on the association rate, as already reported for CO binding to buffalo Hb (Giardina et al., 1992a).

Figure 4 Absorption spectra, in the Soret region, of horse HbNO (a), in the absence (spectrum A) and presence (spectrum B) of 0.1 M InsP_6 , reindeer HbNO (b), chamois HbNO (c) and steinbock HbNO (d)

Absorption spectra of reindeer, chamois and steinbock HbNO were essentially insensitive to InsP_6 . Absorption spectra, in the Soret region, were obtained between pH 6.5 and 7.0, in 0.1 M Mes and Hepes buffer systems plus 0.1 M NaCl, at 20 °C. Spectra were recorded in 1 mm path-length cuvettes. The concentration of HbNO was about 75 μM . The spectra for horse and reindeer HbNO were obtained from the literature (Ascenzi et al., 1990, 1992). For further details, see the text.

The functional data reported here are in keeping with the spectroscopic properties of the nitrosylated derivative in chamois and steinbock Hbs. Figures 3 and 4 show the X-band e.p.r. spectra and absorption spectra respectively of horse HbNO (in the absence and presence of 0.1 M InsP_6) (Ascenzi et al., 1990) as well as of reindeer (Ascenzi et al., 1992), chamois and steinbock HbNO, between pH 6.5 and 7.0, at 100 K. In the absence of allosteric effectors, the X-band e.p.r. spectra of nitrosylated derivatives of several tetrameric Hbs [horse and human Hb

(Perutz, 1979; Blumberg, 1981; Ascenzi et al., 1988, 1990, 1992)] show a rhombic shape and a weak hyperfine pattern in the g_z region (Figure 3a, spectrum A) which have been associated with the high-affinity state of the molecule (Perutz, 1979; Blumberg, 1981). On lowering the pH and/or on addition of allosteric effectors, horse and human HbNO (Perutz, 1979; Blumberg, 1981; Ascenzi et al., 1988, 1990, 1992) undergo a transition toward a species characterized by a spectrum with three-line splitting ($A_z = 1.65$ mT) in the high-magnetic-field region ($g_z = 2.01$) (Figure 3a, spectrum B). Such a pattern is observed in chamois and steinbock HbNO, in the absence and presence of allosteric effectors, similarly to that reported for several ruminant nitrosylated Hbs (e.g. reindeer HbNO) (Ascenzi et al., 1990, 1992) (Figures 3b, 3c and 3d). This e.p.r. spectroscopic feature has been associated with the low-affinity conformation of tetrameric haemoproteins (Perutz, 1979; Blumberg, 1981).

Likewise, the absorption spectra, in the Soret region, of horse and human HbNO display an ϵ value which is dependent on the allosteric effector concentration and pH (Perutz, 1979; Ascenzi et al., 1990, 1992) (Figure 4a). In contrast, the ϵ value for chamois and steinbock HbNO (98 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ and 97 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ respectively at 415 nm) (Figures 4c and 4d) is insensitive to allosteric effectors and corresponds to that obtained for reindeer HbNO, in the absence and presence of allosteric effectors ($\epsilon = 96 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 415 nm) (Ascenzi et al., 1992) (Figure 4b). This absorption spectrum is superimposable on those of horse and human HbNO in the presence of organic (poly)phosphates ($\epsilon = 96 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ and 99 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ respectively at 415 nm) (Perutz, 1979; Ascenzi et al., 1988, 1990, 1992) (Figure 4a, spectrum B). These features suggest that chamois and steinbock HbNO, similarly to that reported for the nitric oxide derivative of opossum, ruminant (reindeer) and turtle Hb (John and Waterman, 1979a, b; Louro and Bemski, 1982; Ascenzi et al., 1990, 1992; Giardina et al., 1992b), is in a low-affinity conformation even in the absence of allosteric effectors (i.e., under the experimental conditions in which the functional data were obtained) (see Figure 1).

Besides the g_z region analysed thus far, the X-band e.p.r. spectra of reindeer (Ascenzi et al., 1992), chamois and steinbock HbNO, in the absence and presence of 0.1 M InsP_6 (see Figures 3b, 3c and 3d), are superimposable on that of human HbNO in the presence of 0.1 M InsP_6 (Perutz, 1979; Ascenzi et al., 1988). On the other hand, these X-band e.p.r. spectra display some differences in the low-magnetic-field region ($g_x = 2.09$) with respect to that of horse HbNO, in the presence of 0.1 M InsP_6

Table 2 Comparison of the amino acid sequences of the N-terminal segments of the α - and β -chains of human, horse, reindeer, chamois and steinbock Hb

Boxes indicate identical residues.

Haemoglobin	α -Chain N-terminus										β -Chain N-terminus									
	NA1	NA2	A1	A2	A3	A4	A5	A6	A7	A8	NA1	NA2	NA3	A1	A2	A3	A4	A5	A6	
Human Hb*	Val	Leu	Ser	Pro	Ala	Asp	Lys	Thr	Asn	Val	Val	His	Leu	Thr	Pro	Glu	Glu	Lys	Ser	
Horse Hb*	Val	Leu	Ser	Ala	Ala	Asp	Lys	Thr	Asn	Val	Val	Gln	Leu	Ser	Gly	Glu	Glu	Lys	Ala	
Reindeer Hb†	Val	Leu	Ser	Ala	Ala	Asp	Lys	Ser	Asn	Val		Met	Leu	Thr	Ser	Glu	Glu	Lys	Ala	
Chamois Hb‡	Val	Leu	Ser	Ala	Ala	Asp	Lys	Ser	Asn	Val		Met	Leu	Thr	Ala	Glu	Glu	Lys	Ala	
Steinbock Hb‡	Val	Leu	Ser	Ala	Ala	Asp	Lys	Ser	Asn	Val		Met	Leu	Thr	Ala	Glu	Glu	Lys		

* From Kleinschmidt and Sgouros (1987).
† From Petruzzelli et al. (1991).
‡ Present study.

Figure 5 Stereoscopic view of the proposed model of apolar interactions occurring at the Gr1(2,3) P_2 -binding site of reindeer Hb, taken as a prototype of ruminant Hb (Ascenzi et al., 1992; Conti et al., 1992)

The hydrophobic pocket formed by Leu(EF2), Leu(EF5) and Val(H11) (B78, B81 and B133 respectively) is in the upper right corner, together with the Met(NA2) (B2) residue.

(Ascenzi et al., 1990) (Figure 3a, spectrum B). John and Waterman (1979b) suggested that differences in the g_x region of the X-band e.p.r. spectra of tetrameric HbNO might reflect differences in the stabilization mode (i.e. in the hydrogen-bonding) of the iron-bound nitric oxide molecule at the distal site of the haem pocket. Thus chamois, steinbock, reindeer and human HbNO might display a common haem-ligand geometry (Deatherage and Moffat, 1979; Ascenzi et al., 1990, 1992), which differs from that of horse HbNO (Ascenzi et al., 1990, 1992).

In ruminant Hb, the lower effects of Gr1(2,3) P_2 and Ins P_6 may be attributed to common amino acid substitutions in the N-terminal segment of the β -chains (i.e., the deletion of Val(NA1), and the replacement of His(NA2), present in human Hb, and Gln(NA2), present in horse Hb, by Met) (Kleinschmidt and Sgouros, 1987; Petruzzelli et al., 1991; Ascenzi et al., 1992; Conti et al., 1992) (Table 2). Thus Met(NA2) and Leu(NA3), which increase the hydrophobic character of the N-terminal region of the β -chains, interact with the central apolar patch formed by the side chains of Leu(EF2), Leu(EF5) and Val(H11) (Figure 5). These intramolecular contacts (Kleinschmidt and Sgouros, 1987; Petruzzelli et al., 1991; Ascenzi et al., 1992; Conti et al., 1992) are likely to maintain ruminant Hb in a quaternary low-affinity conformation even in the absence of allosteric effector; this behaviour is reminiscent of that induced in human and horse Hb by (in)organic (poly)phosphates (Perutz, 1979; Ascenzi et al., 1990, 1992; di Prisco et al., 1991; Conti et al., 1992). The replacement of Pro and Gly in human and horse Hb respectively with Ser in reindeer Hb and Ala in chamois and steinbock Hb (Table 2) at position A2 may have only secondary effects on the stabilization of the low-affinity conformation of the tetramer because it is likely to only partly affect the flexibility of the N-terminal three residues of ruminant β -chains [i.e., Met(NA2), Leu(NA3) and Thr(A1)] (Ascenzi et al., 1992; Conti et al., 1992).

The enhanced effect of Cl⁻ on the functional properties of ruminant Hb (Figure 1 and Table 1) has been related to the presence in the β -chains of these tetrameric O₂-carrying proteins of a specific E8-EF5 hydrophilic region (Kleinschmidt and Sgouros, 1987; Fronticelli, 1990; Coletta et al., 1992), which opens up the possibility of an unusual functionally linked binding site for Cl⁻ (Fronticelli, 1990; Coletta et al., 1992).

Finally, the data reported here allow the following physiological considerations. (i) The increased Bohr effect observed for chamois and steinbock Hb oxygenation compared with arctic ruminant (e.g. reindeer) Hb (Figure 1) may be important for species that are specialized for living at high altitude (the pH of

the blood of chamois and steinbock ranges between 7.2 and 7.5). Hence, the increase in the Bohr effect counterbalances the decrease in O₂ release which, at tissue level, could have resulted from reduced arteriovenous differential gaseous ligand pressure. (ii) The increased overall oxygenation enthalpy, compared with that of arctic ruminant Hb (Figure 2), could be a disadvantage for animals living in a cold environment and it might well be compensated for by the enhanced Bohr effect [the body temperature of chamois and steinbock ranges between 37 °C (for core) and 20 °C (for extremities)], as already demonstrated for reindeer (Klein et al., 1987)]. On the whole, the enhanced pH-dependence of O₂ affinity observed on chamois and steinbock Hbs may be considered to be the best molecular strategy for species that have to survive in extreme environments characterized by both high altitudes and low temperatures.

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